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Biochimica et Biophysica Acta 1758 (2006) 375–383

BIOCHIMICA ET BIOPHYSICA ACTA

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Quantification of the efficiency of cargo delivery by peptidic and pseudo-peptidic Trojan carriers using MALDI-TOF mass spectrometry

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Received 6 October 2005; received in revised form 11 January 2006; accepted 13 January 2006
Available online 8 February 2006

Abstract

We have measured the efficiencies of two novel pseudo-peptidic carriers and various cell-penetrating peptides (Penetratin, $(Arg)_9$ and the third helix of the homeodomain of Knotted-1) to deliver the same cargo inside cells. The cargo that was studied corresponds to the pseudo-substrate of protein kinase C. Cargo delivery was quantified using a recent method based on isotope labeling and MALDI-TOF MS. Results of cargo delivery were compared to the amounts of free CPP internalized inside cells. The third helix of Knotted gave the best results concerning free CPP cellular uptake. It was also found to be the most efficient carrier. This peptide thus emerges as a new CPP with very promising properties.

Keywords: Cell-penetrating peptide; Cargo delivery; Quantification; MALDI-TOF MS

1. Introduction

The study of protein internalization and the identification of the domain responsible for their cellular uptake led to the discovery of the first short Cell-Penetrating Peptides (CPPs) corresponding to Penetratin derived from the *Antennapedia* homeodomain [1] and to Tat-derived peptides [2]. Many other CPPs have been described soon after corresponding to protein transduction domains or consisting of chimeric and synthetic peptides : nuclear localization sequences (NLS) [3,4], Transportan [5], Model Amphipatic Peptides (MAP) [6], polybasic peptides [7,8], amphipathic proline-rich peptides [9], and β-peptides [10]. It has been demonstrated that some CPPs retain the capacity to translocate the plasma membrane even when coupled to hydrophobic or acidic cargoes (e.g., peptides, oligonucleotides, drugs, etc.) [11]. These were named Trojan peptides for their ability to deliver into cells molecules that alone are not internalized efficiently [12]. Different methods have been reported to quantify the CPPs cellular uptake [13]. Most of them are based on the use of peptides labeled by a reporter group such as biotin [1] or a radioisotope [5,14,15]. Fluorescent peptides are also very frequently used and have been quantified by flow cytometry [16], fluorescence correlation microscopy [17], or resonance energy transfer [18]. The results reported for the CPPs cellular uptake efficiencies strongly differ from one study to another. For example, the ratio between the intracellular and extracellular concentrations for Penetratin ranges from 0.6 [17] to 95.0 [19]. The discrepancies observed between studies may be

Abbreviations: Acm, acetamidomethyl; Boc, *tert*-butoxycarbonyl; BSA, bovine serumalbumin; CHCA, α-cyano-4-hydroxycinnamic acid; CHO, Chinese hamster ovary; CPP, cell-penetrating peptide; H-CPP, non-deuterated cell-penetrating peptide; D-CPP, deuterated cell-penetrating peptide; 2Cl-Cbz, 2-chlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DMEM, Dubelcco's modified eagle medium; DIEA, diisopropylethylamine; DMF, *N*, *N*-dimethylformamide; DTT, dithiotreitol; EDTA, ethylenediamine tetraacetic acid; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 1-hydroxy-7-aza-benzotriazole; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MBHA-PS, 4-methylbenzhydrylamine polystyrene; 4-MeOBzl, p-methoxybenzyl; Npys, 3-nitro-2-pyridinesulphenyl; PyAOP, 7-Azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; PKC, protein kinase C; PKCi, proteins kinase C inhibitor; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexa-fluorophosphate; SEM, standard error of the mean; TFA, trifluoroacetamide; TFFH, tetramethylfluoroformamidinium hexafluorophosphate; THF, tetrahydrofuran

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doi:10.1016/j.bbamem.2006.01.012

in part explained by the use of different cell treatments after internalization to distinguish between the internalized and membrane-associated peptides. It has recently been shown that cell washing with buffer is not sufficient to completely remove the membrane-bound peptide [16]. Discrimination between internalized and membrane-associated peptide is often achieved by enzymatic digestion of the non-internalized peptide [20] that may be combined with an heparin treatment [21], by chemical modification [22], or by fluorescence quenching [18,23]. The quantification methods generally give the total amount of internalized CPP or cargo with no possible distinction between the intact and degraded forms. In two cases, fluorescence quantification has been combined with a technique of peptide separation such as HPLC [22] or Cell Activity by Capillary Electrophoresis [24] so that modifications of the CPP or of the cargo could be detected. We have recently reported a method to measure CPP cellular uptake by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [25]. It is based on the combined use of affinity purification of biotinylated CPPs from cell-lysate by streptavidin-coated magnetic beads [26,27] and isotope labeling [28]. This method gives the amount of intact internalized CPP and allows the direct characterization of peptide modifications. It has now been adapted to measure the amount of cargo delivered inside cells by the CPP to study the ability of CPPs to act as Trojan peptides. The cargo that was chosen corresponds to a hydrophilic peptide inhibitor of Protein Kinase C (PKC) [29]. PKC isozymes represent an important pharmacological target since they play key roles in the regulation of cell growth and in pathological developments including cell transformation [30], cancer progression [31] and drug-resistant neoplastic phenotype [32,33]. It has previously been shown that the PKC peptide inhibitor used in this study (PKCi, Fig. 1) does not inhibit protein phosphorylation in cell culture [34,35]. Indeed, despite its cationic character, this peptide is not able to reach the cytoplasm where the PKC isozymes are located. Several strategies have been used for its cellular delivery leading to a fast inhibition of phosphorylation: (1) lipid addition to the cell culture to permeabilize membranes, (2) intracellular injection [36], (3) peptide chemical modifications including N-myristoylation [34,37], N-palmitoylation [38], S-farnesylation [39], and conjugation to a CPP [35]. The present work focuses on the quantification of the delivery of this peptide inhibitor by different Trojan carriers including Penetratin, $(Arg)_9$, the new CPP corresponding to the third helix of Knotted-1 homeodomain (Kno) and two novel pseudo-peptidic carriers (Fig. 1). Results are compared to the amounts of free CPPs internalized inside cells that have been measured previously [25,40].

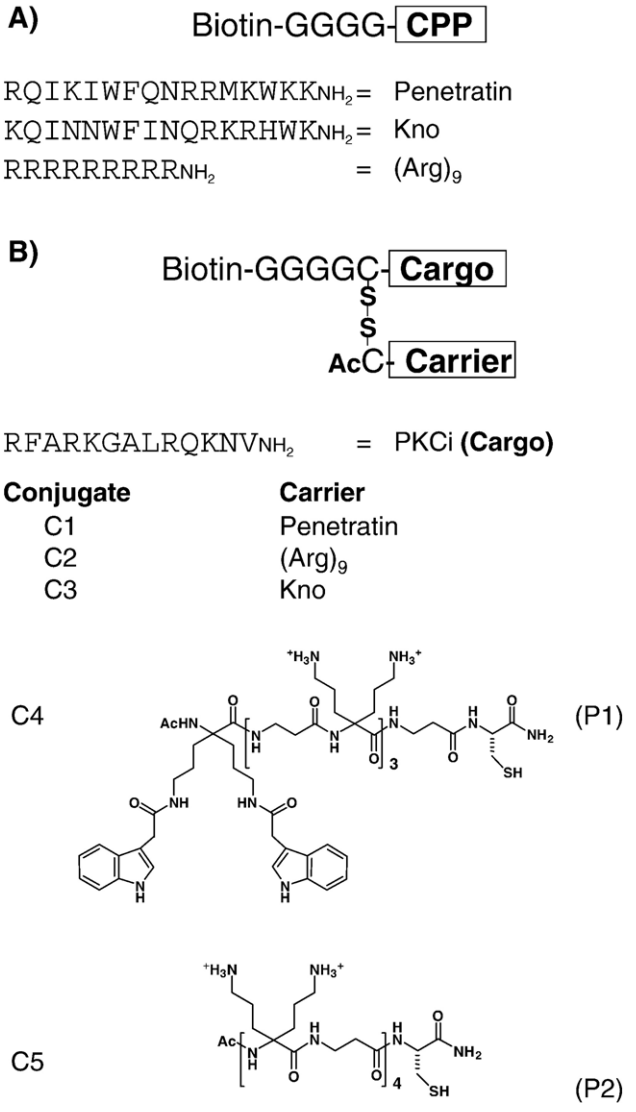

Fig. 1. (A) Construction used for free CPP quantification by MALDI-TOF MS. Internalized CPPs (H-CPPs) are functionalized by an isotope tag composed of four non-deuterated glycine residues, internal standards (D-CPP) are functionalized by four bi-deuterated glycine residues. (B) Construction used for cargo delivery quantification. The internalized species correspond to the conjugates functionalized by four non-deuterated glycine residues. The internal standard corresponds to the free cargo functionalized by four bi-deuterated glycine residues. The sequences of the CPPs, pseudo-peptidic carriers and cargo are indicated.

2. Materials and methods

2.1. Reagents

Standard Boc amino acids, MBHA-PS resin and HBTU were purchased from Senn Chemicals. Solvents (peptide synthesis grade), DCC, HOBt, and PyAOP were obtained from Applied Biosystems. HOAt was purchased from Fluka. [2,2-D_2, 98%]-N-Boc-glycine was obtained from Euriso-top.

2.2. Synthesis of the CPPs

Peptides were assembled by stepwise solid-phase synthesis on a ABI 433A peptide synthesizer (Applied Biosystems) using standard Boc strategy (MBHA-PS resin with a loading of 0.9 mmol NH_2/g, amino acid activation with DCC/HOBt or HBTU) on a 0.1 mmol scale. The peptides were cleaved from the resin by treatment with anhydrous HF (1h30, 0 °C) in the presence of anisole (1.5 mL/g peptidyl-resin) and dimethylsulfide (0.25 mL/g peptidyl-resin) following the standard procedure. They were purified by preparative reverse-phase HPLC on a C8 column, using a linear acetonitrile gradient in an aqueous solution containing 0.1% (v/v) trifluoroacetic acid. Peptides were obtained with a purity > 95%, as assessed by analytical HPLC. They were characterized by MALDI-TOF MS (Voyager Elite, PerSeptive Biosystems) in positive ion reflector mode using the matrix CHCA. Delayed extraction was optimized for 20 kV acceleration voltage

to get the highest resolution in the peptide mass range. The *m/z* of the protonated molecule (first isotope) are given as experimental (theoretical): H-Penetratin, 2699.3 (2699.5); D-Penetratin, 2707.3 (2707.5); H-Kno, 2649.3 (2649.4); D-Kno, 2657.4 (2657.4); H-$(Arg)_9$ 1877.2 (1877.1); D-$(Arg)_9$ 1885.2 (1885.1); D-PKCi-SH, 2108.0 (2108.1); PKCi-SAcm 2156.5 (2156.1).

2.3. Synthesis of the pseudo-peptidic carriers P1 and P2

Amounts of reagent are given in equivalents with respect to the peptidyl-resin unless mentioned otherwise. P_1 and P_2 were synthesized manually using solid-phase Boc strategy on MBHA-PS resin (72 mg, 0.05 mmol). Boc-Cys(4-MeOBzl)-OH and Boc-βAla-OH (10 equiv.) were activated by treatment for 5 min with HBTU (9 equiv.) and DIEA (20 equiv.) in DMF (final concentration of activated amino acid: 0.45 M). Each coupling was allowed to proceed for 30 min under a stream of argon at room temperature. Units U_1, U_2 and U_3 (5 equiv.) were mixed with PyAOP (6 equiv.), HOAt (6 equiv.) and DIEA (20 equiv.) in DMF (final concentration of amino acid 0.29 M) before addition to the peptidyl-resin. Each coupling was allowed to proceed for 12 h at 50 °C under a stream of argon. Double coupling were used. Completion of the reaction was monitored by the Kaiser test. Capping of the unreacted amino groups was performed by treatment of the peptidyl-resin with acetic anhydride (10% in DMF) for 30 min. The Boc protecting group was removed by treatment with trifluoroacetic acid for 3 min. The pseudo-peptide P_1 was synthesized by the consecutive assembly of 3 units U_1 and 1 U_2. The side-chains of U_2 were deprotected with trifluoroacetic acid. 1H-indol-3-acetic acid (20 equiv.) was preactivated for 5 min with HBTU (18 equiv.) and DIEA (40 equiv.) in DMF (final concentration of preactivated acid 0.45 M) and coupled to P1 overnight under a stream of argon at room temperature. The resin-bound polymer P_1 was washed with THF, suspended in 18 mL of a mixture of NaOH (2 M) / THF (1:2, v/v) and stirred for 24 h to remove the TFA protecting groups. The resin was washed several times with a mixture of water and THF and with pure THF. The pseudo-peptide P_2 was assembled from 3 units U_3 and 1 U_2. The protecting groups of the side-chains of U2 were removed with trifluoroacetic acid before HF clivage. Cleavage of the polymers P1 and P2 from the solid support was carried out by treatment with HF (2 h, 0 °C) in presence of anisole (1.5 mL/g peptidyl-resin), dimethylsulfide (0.25 mL/g peptidyl-resin) and *p*-thiocresol (300 mg/g peptidyl-resin). After HF removal under vacuum, the peptide was precipitated in cold diethyl ether and dissolved in a degassed aqueous solution of acetic acid (10%). Polymers P_1 and P_2 were freeze-dried.

2.4. Synthesis of the carrier-cargo conjugates via disulfide bridge

The crude polymers P1 and P2 were dissolved in a degassed solution of 10% acetic acid and mixed with a slight excess of peptide PKCi-Cys(Npys) (about 1.5 equiv.). The reaction was followed by HPLC and conjugates were purified by reverse-phase HPLC on a C8 column, using a linear gradient of acetonitrile in an aqueous solution containing 0.1% (v/v) trifluoroacetic acid. The same procedure was used to synthesize the CPP-cargo conjugates from the purified CPP-Cys and PKCi-Cys(Npys) peptides. Conjugates were characterized by MALDI-TOF MS in positive ion reflector or linear mode using the CHCA matrix. For the protonated conjugates at $m/z<3600$, the first isotope was detected: C4 (PKCi-S-S-P1) 3543.0 (3541.9); C5 (PKCi-S-S-P2) 3228.1 (3227.8). For the protonated conjugates at $m/z>3600$, the average *m/z* was determined: C1 (PKCi-S-S-Penetratin), 4490.0 (4489.4); C2 (PKCi-S-S-$(Arg)_9$) 3667.1 (3666.4); C3 (PKCi-S-S-Kno) 4442.1 (4439.3). The *m/z* values are given as experimental (theoretical).

2.5. Measure of the cellular uptake by MALDI-TOF MS

The internalization experiments were performed using 12-well plates. In each well, 10^6 adherent CHO K1 cells were incubated for 75 min at 37 °C with 1 mL culture medium (DMEM) containing the biotinylated non-deuterated CPP (H-CPP) or the conjugate (H-PKCi-S-S-Carrier) (7.5 μM). The cells were then washed 3 times with 2 mL culture medium, treated for 3 min at 37 °C with 500 μL of a solution containing 0.05% trypsin, 0.02% EDTA and transferred at 4 °C. Soybean trypsin inhibitor (100 μL, 5 mg/mL) and BSA (100 μL, 1 mg/mL) were added. The cell suspension was transferred in a 1.5-mL conic tube and the well was washed with 500 μL of 50 mM Tris–HCl buffer (pH 7.4). Both suspensions were pooled and centrifuged for 2 min at 3000 rpm. The pellet containing the intact cells was washed with 1 mL of 50 mM Tris–HCl buffer (pH 7.4), 0.1% BSA (buffer A) and centrifuged again. The pellet was mixed with a known amount of the adequate biotinylated deuterated standard (D-CPP or D-PKCi-SH) and 150 μL of a solution containing 0.3% Triton X100 and 1 M NaCl. The mixture was heated for 15 min at 100 °C. The cell lysate was then centrifuged for 5 min at 10,000 rpm at 4 °C. The supernatant was mixed with 850 μL of buffer A in the case of CPP quantification or buffer A containing 2 mM DTT in the case of cargo (PKCi) quantification. The mixture was incubated for 2 h at 4 °C with 100 μg streptavidin-coated magnetic beads (Dynabeads M-280, Dynal, France) to capture the biotinylated peptides. After bead immobilization with the Dynal magnetic particule concentrator, the supernatant was removed. Beads were washed twice with buffer A (200 μL), twice with buffer A containing 0.1% sodium dodecylsulfate (200 μL), twice with buffer A containing 1 M NaCl (200 μL) and with water (2 × 200, 100, 3 × 50 and 10 μL). Beads were mixed with 3 μL of a saturated solution of CHCA in acetonitrile/water–0.1% trifluoroacetic acid (4:1) and 1 μL of the mixture was deposited on the MALDI-TOF sample holder. The samples were analyzed by MALDI-TOF MS (ion positive reflector mode) on a Voyager Elite PerSeptive Biosystems mass spectrometer. The laser fluence used was near the threshold of peptide ions production for the considered deposits (bead-associated peptide covered with CHCA matrix). Spectra were averaged from several hundred laser shots recorded on different spots of the deposit to get an isotope pattern similar to that expected. The area of the $[M+H]^+$ signals including all the isotopes of the intact non-deuterated species (H-CPP or H-PKCi-SH) and deuterated species (D-CPP or D-PKCi-SH) were measured. The amount of internalized species was calculated from the areas' ratio. In the first experiment, the amount of internalized peptide was evaluated for each CPP or conjugate by adding various and known amounts of the adequate standard to samples containing the internalized peptide. The amount of standard added in the subsequent experiments was chosen to get a peptide ratio close to 1:1 to allow a precise quantification. The internalization experiments were performed in duplicates and repeated at least four times independently. To quantify the internalization of the free cargo H-PKCi-SAcm, a known amount D-PKCi-SH was added to the sample before cell lysis, the lysate was then mixed with 850 μL of buffer A containing 2 mM DTT and 20 mM iodoacetamide and incubated for 2 h at 4 °C with the streptavidin-coated magnetic beads.

3. Results

3.1. Synthesis of the pseudo-peptidic cationic carriers P1 and P2

Two new pseudo-peptidic carriers P1 and P2 incorporating in their sequence the α,α-disubstituted amino acid bis-ornithine (2,2-bis (aminopropyl)glycine) have been synthesized (Fig. 1). Consecutive bis-ornithine residues are separated in the polymers by a β-alanine. P1 is functionnalized by six amine and two indol groups whereas P2 contains eight amine groups. Indols were introduced in P1 in an attempt to increase the affinity of the carrier for the cellular membrane.

The different units U1 to U3 (Fig. 2) containing the residue bis-ornithine were prepared in solution. (Aussedat, B, unpublished data). These units were protected differently on the side-chains of the bis-ornithine in order to allow the future preparation of a small library of polymers with various amine functionalizations. Polymers P1 and P2 were synthesized on solid support from units U1 to U3 after optimization of the coupling conditions. We first tested the activation of the dipeptidic units by HBTU, HATU, PyBOP or TFFH. In each case, the activated ester was reacted for 12 h at 50 °C with the elongating polymer and double or triple couplings were performed. The best result was obtained with HATU but the

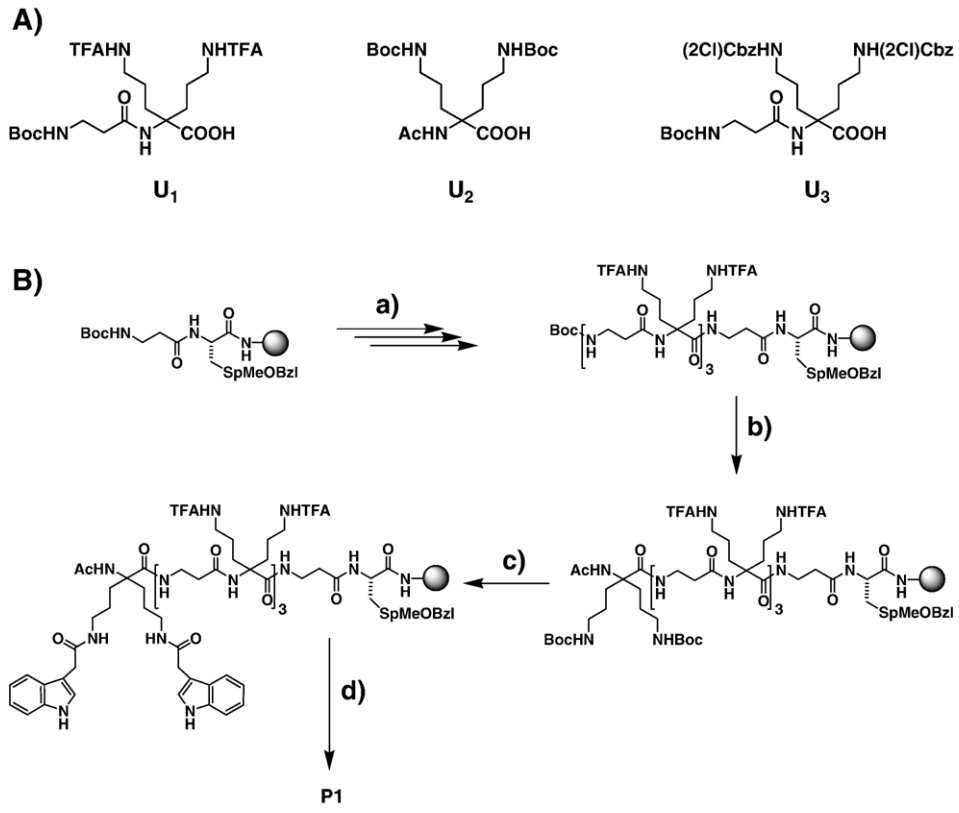

Fig. 2. (A) Units containing bis-ornithine. (B) Synthesis of the pseudo-peptidic carrier P1. (a) trifluoroacetic acid 3 min then double coupling of U1 activated in situ by PyAOP/HOAt/DIEA in DMF, 50 °C for 12 h (3 cycles). (b) trifluoroacetic acid 3 min then double coupling of U2 activated in situ by PyAOP/HOAt/DIEA in DMF, 50 °C for 12 h. (c) trifluoroacetic acid 3 min then coupling of 1H-indol-3-acetic acid activated by HBTU/DIEA in DMF, 12 h room temperature. (d) NaOH/water/THF then HF cleavage.

coupling reactions remained incomplete. When the dipeptides activated in situ with a mixture of PyAOP and HOAt were reacted for 12 h at 50 °C with the polymer and double couplings were used, couplings were found to be almost complete (no aborted polymer detected after cleavage from the support). P1 was synthesized by consecutive coupling of three units U1 protected on the side-chains by a trifluoroacetamide group and one unit U2 protected by Boc (Fig. 2). Indol groups were introduced on the side chain of the N-terminal unit after Boc removal (Fig. 2). Two major compounds were detected in the crude mixture after cleavage of the polymer from the resin. These were separated by HPLC and characterized by MALDI-TOF MS. The major compound was found to be the expected product P1 whereas the side-product was identified as the polymer containing a dehydro-alanine instead of a cysteine at the C-terminus. The formation of dehydro-alanine probably took place during the removal of the trifluoroacetamide groups using stringent basic conditions. Although, the formation of this side-product may be avoided by optimizing the deprotection conditions (use of shorter reaction times), we chose to change protecting group in the subsequent polymer synthesis. P2 was assembled from units U3 and U2. After cleavage from solid support by treatment with HF, the crude polymers containing a free cysteine were conjugated in solution to the PKCi cargo containing a cysteine activated by a 3-nitro-2-pyridinesulfenyl (Npys) group. Conjugates were purified by reverse phase HPLC.

3.2. Quantification of the cellular uptake by MALDI-TOF MS

The measure of cellular uptake of free CPPs has been performed using the method based on MALDI-TOF MS recently described by our group [25]. Peptide quantification by MALDI-TOF MS is achieved by using an internal standard corresponding to a peptide with the same sequence but labeled with a stable isotope. The CPPs have the general structure represented in Fig. 1A. They are functionalized on their N-terminus by biotin plus an isotope tag composed of four non-deuterated glycine residues for the internalized species (H-CPP) and four bi-deuterated glycine residues for the standard (D-CPP). A known amount of standard is added at the end of the incubation of the H-CPP with cells before lysis. The biotinylated peptides are extracted from the lysate, concentrated and desalted using streptavidin-coated magnetic beads to allow the analysis by MALDI-TOF MS. Because both H-CPP and D-CPP have the same sequence, they have the same affinity for the streptavidin-coated beads and exhibit the same efficiency of desorption/ionisation by MALDI-TOF MS. Therefore, the relative intensity of their signals on the mass spectra corresponds to their relative proportion in the sample. The amount of intact internalized peptide is calculated from the ratio between the peak areas of H-CPP and D-CPP ($[M+H]^+$ peak of the intact peptides, Fig. 3A). Because of the non-complete deuteration of the D-CPPs (typically 98% of the peptides contain 8 deuteriums and 2% contain 7 deuteriums) and

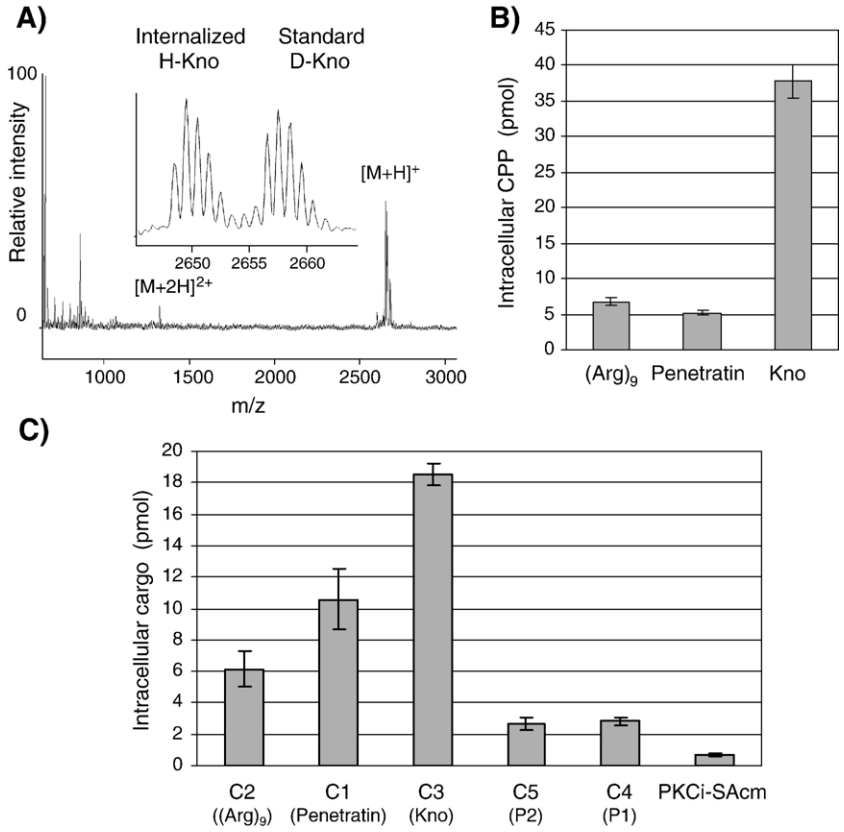

Fig. 3. (A) MALDI-TOF mass spectrum obtained for the cellular uptake of Kno. The peaks at $m/z < 1000$ correspond to the matrix or polymer covering the streptavidin-coated magnetic beads. The insert shows the $[M+H]^+$ peaks of the intact non-deuterated (H-Kno) and deuterated peptide (D-Kno). (B) Total amount of intact internalized free CPP in 10^6 CHO cells. (C) Total amount of intact internalized PKCi in 10^6 CHO cells for the different conjugates (carriers are indicated under brackets). Each data is the average result of at least three independent experiments performed in duplicates or triplicates ± S.E.M.

fluctuations of the isotopes intensity between mass spectra, the quantification is based upon the sum of the peaks area of all isotopes for both H-CPP and D-CPP (including the D7 and D8 components) after background subtraction. Similar results are observed using peaks height instead of peaks area. We have also observed that using the sum of the peaks area of all isotopes gives a lower scattering in the quantification compared to using the peak height of the first or the most abundant isotope.

Using this method, we had previously measured the amount of internalized free CPP in CHO cells after 75 min incubation at 37 °C at a concentration of 7.5 μM (Fig. 3B). Kno gave the highest amount of intact intracellular peptide with 37.8 ± 2.3 pmol (mean value ± S.E.M., $n = 8$) in 10^6 cells [40]. This corresponds to an estimated intracellular concentration of 25.2 ± 1.5 μM based on a volume per cell of 1.5 pL. $(Arg)_9$ and Penetratin gave intracellular concentrations of 4.5 ± 0.4 μM ($n = 8$) and 3.4 ± 0.2 μM ($n = 12$), respectively [25]. No intense signals corresponding to an intracellular degradation have been detected for these CPPs.

The method has been adapted here to measure the amount of cargo delivered inside cells by a carrier. For this purpose we designed the construction presented in Fig. 1B in which the cargo is functionalized by the biotin and the isotope tag and is linked via a disulfide bridge to the carrier. The cargo is expected to be released from the carrier after disulfide bond reduction inside cells [41,42]. The species quantified by MALDI-TOF MS in this experiment thus corresponds to the free cargo and the free deuterated cargo must be used as internal standard. The cargo studied here is the peptide PKCi which is an inhibitor of protein kinase C (Fig. 1B). The first internalization experiment was performed with the conjugate PKCi-S-S-Penetratin (C1) using the protocol that was developed previously to quantify the free CPP cellular uptake. We then observed on the mass spectra the signals of non-deuterated and deuterated PKCi-SH as well as less intense signals corresponding to PKCi-S-S-glutathione conjugates. No signal corresponding to the conjugate C1 was detected. It was found that both deuterated and non-deuterated PKCi-SH peptides had reacted with glutathione to the same extent showing that adduct formation occurred after cell lysis most certainly during the step of peptide capture by streptavidin-coated beads. To avoid the formation of adducts with glutathione that reduce the intensity of the signal of the quantified species, DTT was added to the sample during peptide recovery. The amount of PKCi delivered inside cells by the three different CPPs (Penetratin, Kno and $(Arg)_9$) and the pseudo-peptidic carriers P1 and P2 was measured using the protocol described in Fig. 4. It was compared to the uptake of the free PKCi. To avoid the dimerization of the free cargo H-PKCi-SH during incubation with cells, the sulfhydryl group was reacted with iodoacetamide to give the peptide H-PKCi-SAcm. The corresponding

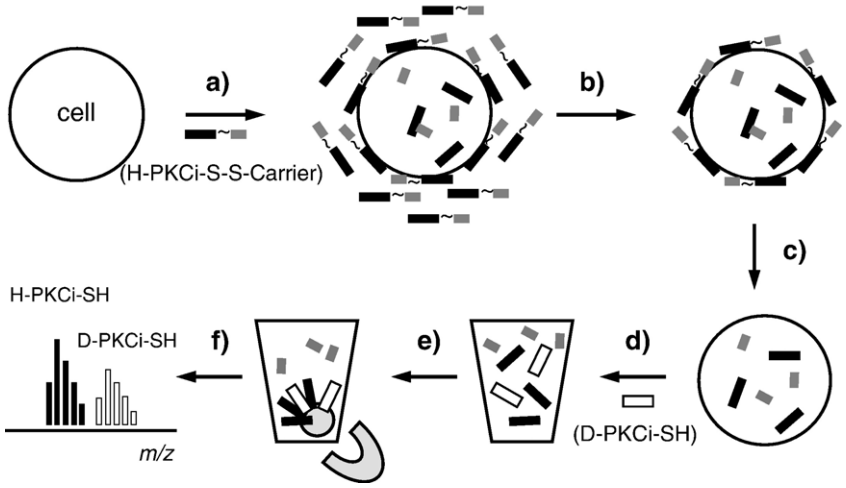

Fig. 4. Quantification of the internalized PKCi cargo by MALDI-TOF MS. (a) Cells are incubated with the conjugate. (b) Cells are washed. (c) Membrane-bound conjugate is digested by trypsin then trypsin inhibitor is added. (d) A known and adequate amount of internal standard is added, cell lysis is performed in 0.3% Triton X100, 1 M NaCl and the sample is heated for 15 min at 100 °C. (e) Biotinylated peptides are captured by streptavidin-coated magnetic beads in presence of DTT. (f) Beads are washed and analyzed by MALDI-TOF MS. The ratio between the MH^+ peak areas of H-cargo-SH and D-cargo-SH yields the absolute amount of internalized cargo.

deuterated standard was formed by adding iodoacetamide in the sample during peptide capture by the streptavidin-coated beads. No D-PKCi-SH was observed in the mass spectra showing that in situ alkylation of the cysteine was complete. The free cargo or the cargo-carrier conjugates were incubated at a concentration of 7.5 μM for 75 min at 37 °C. In these conditions, the amount of free cargo internalized in 10^6 CHO cells was found to be 0.7±0.1 pmol (Fig. 3C). The amount of intact intracellular PKCi was enhanced 4 times compared to the reference when the PKCi peptide was delivered by the pseudopeptides P1 and P2 and up to 26 times when it was delivered by Kno (Fig. 3C). When Kno was used as carrier, the estimated intracellular concentration of intact PKCi was found to be 12.3±0.5 μM. No intense signals corresponding to an intracellular degradation of PKCi were detected in the mass spectra.

4. Discussion

4.1. Methods for the quantification of the CPP cellular uptake and cargo delivery

Most of the methods described in the literature to measure the CPPs cellular uptake give the total amount of internalized peptide with no distinction between the intact and degraded forms [13]. We have recently reported a method to quantify the uptake of CPPs based on MALDI-TOF MS [25]. The use of MALDI-TOF MS allows direct characterization of the different species present in the sample. This gives different advantages. The method provides the amount of intracellular intact CPP. Distinction between the internalized peptide and the peptide bound to the cell surface can be achieved by modification of the extracellular peptide. In our protocol the membrane-bound peptide is digested by trypsin. Digests are expected to have a reduced affinity for the cell membrane and to be removed by washing. Even if some of the peptide fragments retain affinity for the membrane and are not eliminated, they can still be distinguished from intracellular intact peptide by their molecular weight. As demonstrated earlier, the use of MALDI-TOF MS also allows the direct comparison of the uptake efficiencies of different peptides incubated together for screening experiments [25]. Finally, it potentially enables the study of CPP modifications occurring inside cells such as degradation. MALDI-TOF MS is not a quantitative technique. Peptide quantification can only be achieved by using an internal standard which consists of a peptide with the same sequence and labeled with a stable isotope. In our method to quantify the uptake of free CPPs, the CPP is functionalized by an isotope tag containing four non-deuterated or bi-deuterated glycine residues. The method gives the total amount of CPP protected against extracellular trypsin digestion which is assimilated to intracellular peptide. This amount should result from the CPP internalization and its potential intracellular degradation. Only trypsin-sensitive carriers can be studied with this protocol. For non-peptidic carriers, the discrimination between intracellular and membrane-associated peptides would be problematic.

In this study, the technique of quantification by MALDI-TOF MS has been adapted to measure the amount of cargo delivered inside cells by a carrier. To enable its quantification, the cargo is functionalized by the isotope tag previously described. It is linked to the carrier via a disulfide bridge (Fig. 1B). With this construction, it is now the capacity of a CPP to act as a Trojan carrier that is studied. The cargo that was chosen corresponds to a peptide inhibitor of protein kinase C (Fig. 1B). This peptide contains several basic residues in its sequence so that distinction between the internalized and the membrane-bound peptide can still be performed by trypsin digestion. This new method is used to compare the efficiency of different carriers to transport the same cargo. It is very useful for the study of non-peptidic carriers because it solves the problem of discrimination between the internalized and membrane-bound

species. Since, in this case, it is always the same species that is quantified, the quantification sensibility is similar in all experiments and most importantly problems related to differences of accessibility or sensitivity to trypsin of the membrane-bound peptide are avoided. This method gives the amount of intact intracellular cargo resulting from its delivery and its potential intracellular degradation. The intracellular stability of the cargo most probably depends on the carrier used since it can address the cargo to different intracellular compartments. This method thus gives important information for the choice of the best carrier since only the intact cargo will provide the expected biological activity.

4.2. Comparison of the efficiencies of PKCi delivery by various carriers

It was found here that the free PKCi peptide (PKCi-SAcm) is internalized in CHO cells but with very low efficiency. However, all the CPPs and pseudo-peptides that were conjugated to PKCi increased the cargo intracellular concentration showing that they all behave as carriers. Pseudo-peptidic carriers P1 and P2 gave very similar results showing that the loss of positive charges can be compensated by the addition of indol groups. P1 and P2 are functionalized by ammoniums, which have been shown in the literature to be less efficient than guanidiniums to promote membrane translocation [43]. Guanidylation of the bis-ornithine side-chains may enhance the pseudo-peptides cellular uptake. The CPPs $(Arg)_9$, Penetratin and the third helix of Knotted were found to be more efficient to deliver PKCi. Kno gave the best result and was found to be the only carrier leading to an intracellular concentration of intact cargo (12.3 ± 0.5 μM) higher than the concentration of conjugate applied outside cells (7.5 μM).

Previous experiments performed with neurons [35] or human foreskin fibroblasts [34] have shown that the unconjugated PKCi peptide had no biological effect probably because it cannot reach its cytosolic target. Conjugation of PKCi to Penetratin led to 80% inhibition of the phorbol ester-stimulated PKC in neurons [35]. It will now be interesting to see if the same effects are observed in CHO cells and correlate the biological data with the quantification results. This will indicate whether the PKCi internalized alone or conjugated to the different Trojan carriers is able to reach its cytosolic target or if it remains trapped in cellular compartments such as endosomes.

4.3. Comparison between the efficiencies of free CPP uptake and cargo delivery

Two types of experiments have been done to characterize $(Arg)_9$, Penetratin and Kno: the quantification of free CPP cellular uptake and the measure of cargo internalization when conjugated to a carrier (comparison of data presented in Fig. 3B and 3C). In the case of $(Arg)_9$, the intracellular amounts of CPP and cargo were similar. In contrast, the amount of cargo delivered by Penetratin was higher than the amount of free Penetratin internalized. The opposite result was obtained for Kno. The differences observed when measuring the internalized conjugated cargo or the free CPP suggest an effect of the cargo attachment to the CPP on the efficiency of uptake. In the case of Penetratin there might be a positive cooperative effect between the CPP and the cargo for internalization. Another possible explanation is that the differences observed between both type of experiments reflect different rates of intracellular degradation for the cargo and the CPP. Indeed, in both cases, it is the amount of intact species that is measured. No intense signals of intracellular digests have been detected on the mass spectra for the CPPs or the PKCi. However, it cannot be excluded that a fraction of the internalized peptides has been completely digested and is not detected by MALDI-TOF MS or that the degraded fragments have leaked out of the cells. The free CPP and the CPP-cargo conjugate may enter cells via different pathways and may be addressed to different compartments leading to different degradation rates. The study of conjugates with the cargo and the carrier both labelled with the isotope tag and the biotin may help answering these questions.

5. Conclusion

We have reported here a new method to quantify the efficiency of cargo delivery based on MALDI-TOF mass spectrometry. The cargo that was chosen corresponds to a trypsin-sensitive peptide allowing the easy and accurate distinction between the internalized and membrane-bound cargo. This method present the major advantage to be applicable to the study of peptidic as well as non-peptidic carriers. Two new pseudo-peptidic carriers incorporating in their sequence the α,α-disubstituted amino acid bis-orthinine have been examined here. Work is in progress to study the effect of different functionalization of the side-chains of this amino acid on cellular uptake. Finally, the efficiencies of cellular uptake and cargo delivery of three CPPs have been compared. The peptide corresponding to the third helix of Knotted-1 homeodomain appears as a new promising Trojan peptide exhibiting an improved efficiency of cargo delivery compared to the already widely used CPPs Penetratin and $(Arg)_9$. It will now be interesting to evaluate the biological activity of the cargo when delivered by these different carriers and to analyze the cytotoxicity of the carriers.

Acknowledgments

This study was supported in part by grants from the Conseil Régional D'Ile de France, from FNS and CNRS (ACI "Dynamique et Réactivité des Assemblages Biologiques" 04/030) and from INTAS (International association for the promotion of co-operation with scientists from the new independent states of the former Soviet Union).

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